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Acute Toxicity of Four Drinking Water Disinfection By-Products to Japanese Medaka Fish

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Drinking water disinfection is necessary to protect public health. Successful disinfection practices over the past century have virtually eliminated drinking water microbial disease threats through the use of chemical oxidant disinfectants such as chlorine, chloramines, chlorine dioxide, and ozone (Fawell et al. 1997). For each disinfection process, there are issues of cost and effectiveness, as well as issues of chemical by-products from the reaction of chemical oxidants with naturally occurring organic and inorganic substances in the source water.

Research has begun to more extensively characterize the toxicity of drinking water disinfection by-products (Boorman et al. 1999). Trihalomethanes, such as chloroform and bromodichloromethane (BDCM), are the most frequently found constituents of drinking water disinfection (Borum et al. 1998, Krasner et al. 1989). Dibromoacetic acid (DBAA) is a member of the haloacetic acid family of chemicals, another frequently found disinfection by-product (IARC 1991). Chlorate is a frequently occurring by-product of chlorine dioxide disinfection (ILSI Report 1998). Public utility summary reports of measurements have characterized the amounts of disinfection by-products in finished water. Median values of chloroform, BDCM, DBAA, and chlorate in drinking water have been reported as 22.4, 6.7, <0.25, and 230 ug/L, respectively (Borum et al. 1998).

Nonmammalian aquatic models, such as the Japanese medaka (*Oryzias latipes*), offer the ability to do large-scale bioassays and directly assess chemical effects in an aqueous media. The medaka model has been used extensively in both acute and chronic toxicity studies (Powers 1989, Hatanaka et al. 1982, Walker et al. 1985, Gardner et al. 1990, Toussaint et al. 1999). Due to the animal's small size and ease of laboratory culturing, same age test groups are obtained easily. This paper describes the acute toxicity of these four disinfection by-product chemicals to medaka. Fish acute toxicity levels will be compared to drinking water concentration levels and will be used to determine medaka dosing concentrations for chronic testing of these chemicals.

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MATERIALS AND METHODS

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Academy Press, Washington, DC, 1996, in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Medaka fish were obtained from in-house culture facilities. Fish were reared as described previously (Toussaint et al. 1999), and 14 ±1day old fish were selected for testing. A hard groundwater was processed through a softener, reverse osmosis, blended with raw groundwater, carbon filtered, particle filtered, and sterilized with ultraviolet light before being used as the dilution water. The tests were conducted within mesh sided polypropylene cylinders (water column = 9 cm x 17 cm; mesh = 32 x 32 lines/inch) immersed in 5 gallon glass aquaria that were sealed with glass hinged tops. Water temperature was maintained at 25±1 °C. Fish were held under a light/dark cycle of 16/8 h. Test solution was replenished in the aquaria by the proportional diluter every 6 min 30 sec ±30 sec at a rate of 300 ± 15 mL per cycle throughout the 96 h test. Water quality was monitored on the first, middle, and last day of exposure in one replicate each of the control, low, mid, and high concentration tanks. Temperature, pH, conductivity, and dissolved oxygen were measured with a Cole Parmer Water Analyzer Model A51500. Alkalinity and hardness were measured in controls only by potentiometric titration and EDTA titrimetric methods, respectively. Light intensities averaged 733 lux.

Few literature citations for fish exposures to drinking water disinfection by-products were available to aid in concentration selection, so logarithmically spaced range finding concentrations of 0, 0.1, 1, 10, 100, and 1000 mg/L were chosen. Each range finding test had 10 fish per replicate and 4 replicates per treatment, 240 fish total per range finding test. All animals and exposure vessel positions were randomized. Each range finding test was a one-way treatment structure in a randomized complete block design structure with nested replication. With the exception of chlorate, all chemical concentrations during range finding tests were measured three times during the start, middle, and end of the test. Nominal chlorate concentration values were used for the range finding test pending completion of the refinement of the chlorate chemical analysis method. Since BDCM is similar chemically to chloroform, no range find testing was done with BDCM. Chemical concentration selections for the definitive tests were based on range finding results. Disinfection by-product levels in the dilution water were below detection levels.

Each definitive test was a one-way treatment structure with a randomized complete block design structure with nested replication. There were six treatment levels including a control and four replicates for each chemical tested. After range finding tests bracketed the range to be tested, a 0.6 dilution series was used to

establish chemical concentrations. Two five gallon aquaria housed two replicate containers for each chemical and control treatment level. Ten fish were used per replicate, for a total of 240 fish used per definitive test. The fish survival endpoint was analyzed by probit analysis using SAS PROC PROBIT computer software (SAS, 1989). Fish length and weight endpoints were analyzed by analysis of variance (ANOVA) and regression analysis using SAS PROC GLM computer software (SAS, 1989).

Chloroform (CAS # 67-66-3) and bromodichloromethane (CAS # 75-27-4), both of 98+% purity, were obtained from Aldrich Chemical Company, Milwaukee, WI. Stocks of 4 g/L were prepared for both chloroform and BDCM in sealed glass carboys with mixing for 24 h prior to use. Daily stock samples (40 mL in glass head space vials) were refrigerated until analysis. Samples were analyzed using a Hewlett Packard 6890 gas chromatograph (GC) equipped with a flame ionization detector and interfaced to a Hewlett Packard model 7694 headspace sampler. The capillary column used throughout the study was a Hewlett Packard 25 m HP-1 (cross-linked methyl polysiloxane) 0.2 mm id and 0.33 μm film thickness. The GC oven temperature was 40°C, the isothermal inlet was 250°C, and the electron capture detector was maintained at 300°C.

To analyze the required number of chloroform and BDCM samples in a timely manner, a static headspace sampling method was developed. Headspace sampling is a reliable method for sample introduction into a GC that is rapid, reproducible (Rothweiler, 1994), and sensitive (Parsons and Stafford, 1994). Since there were no sample matrix interferences in this analysis, a minimum of sample preparation was required. Three 5 mL aliquots of each sample were placed in 10 mL headspace vials and sealed with teflon lined crimp caps. The vials were placed in the headspace sampler with the sampler oven set at 60°C, the 1 mL sample loop at 65°C, and the transfer line to the GC set at 70°C. The sample vial equilibration time was 30 minutes with agitation set on high. The detection limit for chloroform and BDCM was 3 mg/L. The average percent recoveries for chloroform and BDCM were 94% and 99%, respectively.

Sodium chlorate (CAS # 7775-09-9) was obtained from Aldrich Chemical Company, Milwaukee, WI. Stock solutions of 64 g/L chlorate were prepared in a glass carboy by stirring for 24 h prior to use. Samples were analyzed using a Dionex 500 series Ion Chromatograph (IC) equipped with conductivity detector, autosampler, anion self generating suppressor ASRS-1 4 mm and Dionex Peak Net Data system. A Dionex IonPac® AS14 4X250 mm column with an IonPac® AG14 guard column was used for the separation. Dionex method 5.9 Isocratic analysis of selected oxyanions was used to determine chlorate concentrations (Dionex, 1996). Briefly, the mobile phase consisted of 2.7 mM sodium carbonate / 1 mM sodium bicarbonate buffer in reagent grade water. A flow rate of 1.2 milliliters / minute was used. The injection volume was 10 microliters. Samples (10 mL each) were collected in Nalgene plastic 60 mL bottles. The detection limit

was 10 mg/L. The average percent recovery for chlorate was 99%.

Dibromoacetic acid (DBAA) (CAS #631-64-1) was obtained from Aldrich Chemical Company, Milwaukee, WI. Stock solutions of 18 g/L DBAA were prepared in a glass carboy by stirring for 24 h prior to use. Samples were analyzed using a Hewlett Packard 1050 series HPLC equipped with variable wavelength detector and autosampler. A Supelco C-18 column 25 cm X 0.46 cm, 5 micron particle size equipped with a Waters C-18 guard column was used for the separation. The mobile phase consisted of 2.5% acetonitrile and 97.5% of 0.2% phosphoric acid with a flow rate of 1.5 milliliters / minute. The effluent was monitored using an ultraviolet detector monitoring 200 nm. The injection volume was 20 microliters. This method was developed by USACEHR specifically for the analysis of DBAA at the levels being tested. Ten mL samples were collected in 40 mL glass scintillation vials and refrigerated until analysis. The instrumental detection limit for DBAA was 4 mg/L. The average percent recovery for DBAA was 98%.

RESULTS AND DISCUSSION

Increasing chemical concentration had selective effects on water quality parameters. DBAA lowered pH while sodium chlorate significantly raised conductivity (Table 1). The dissolved oxygen levels in the highest chloroform concentration were depressed but were above 60% saturation. Dilution water alkalinity and hardness were 138 and 187 mg/L $CaCO_3$, respectively.

Mean measured concentrations for definitive testing with all four chemicals with pooled replicates averaged over three timepoints are shown in Table 2. With one exception, all chlorate and DBAA concentrations were within 10% of nominal values. The two volatile chemicals, BDCM and chloroform, were most often 20-30% of nominal concentrations.

The 96 hr LC50s and their corresponding 95% fiducial limits for chloroform, BDCM, chlorate, and DBAA are given in Table 3. Only an LC50 range was obtained for DBAA. Adverse effects on fish length and weight (ANOVA $p<0.05$) were observed at the lowest concentrations of each chemical tested, therefore, NOELs were estimated (when possible) using regression analysis. Based on this information, medaka sensitivity to the disinfectant by-products tested appeared to be ordered from most to least sensitive as bromodichloromethane>chloroform> dibromoacetic acid>chlorate.

During range finding experiments with DBAA, all the fish at the highest nominal concentration, 1000 mg/L DBAA, died within two hours of exposure. The pH for the 1000 mg/L exposure was found to be 3.4. Dilutions of the 1000 mg/L stock were made and 400 mg/L DBAA was found to have a pH of 6.3-6.5. Concentrations higher than 400 mg/L (600 and 800 mg/L) had pH's of 4 or below.

Table 1. Mean water quality parameters for control and the high concentration of four drinking water disinfection byproducts.

Parameter	Control	$CHCl_3$ 328 mg/L	BDCM 251 mg/L	Chlorate 8309 mg/L	DBAA 393 mg/L
(# of measurements)	(12)	(3)	(3)	(3)	(3)
Temperature (°C)	25.0	25.5	25.1	25.2	25.0
pH[a] (standard units)	7.7-7.9	7.6-7.7	7.8-7.9	7.9-8.0	6.3-6.5
Dissolved Oxygen (mg/L)	7.5	5.7	7.9	7.0	7.5
Conductivity (µmhos/cm)	637	622	623	10,300	544
Un-ionized ammonia (mg/L)	0.001	0.001	0.001	0.001	0.001

$CHCl_3$=chloroform, BDCM=bromodichloromethane, DBAA=dibromoacetic acid
[a] pH values are ranges

Table 2. Mean measured concentrations (mg/L) for disinfectant by-product single chemical testing. Each measured value is the mean of 6 analyses.

Chemical	Value	Test Concentrations in mg/L					
$CHCl_3$	nominal	0	32	54	90	150	250
	measured	<3	35	56	105	172	328
	SE	0	1	2	4	7	9
BDCM	nominal	0	45	76	126	210	350
	measured	<3	29	51	82	146	251
	SE	0	2	4	6	10	17
Chlorate	nominal	0	1037	1728	2880	4880	8000
	measured	<10	1263	1738	2675	4776	8309
	SE	0	240	49	112	58	228
DBAA	nominal	0	52	86	144	240	400
	measured	<4	49	80	142	231	393
	SE	0	2	8	5	8	12

$CHCl_3$=chloroform, BDCM=bromodichloromethane, DBAA=dibromoacetic acid
SE = standard error

Table 3. Medaka 96 h LC50 and NOEL range estimates for four drinking water disinfection by-products.

Chemical	96 h LC50[a]		NOEL Range[b]	
	Estimate (mg/L)	95% Fiducial Limits (mg/L)	Length (mg/L)	Weight (mg/L)
Chloroform	138	129, 149	12-15	14-18
BDCM	72	56, 83	5-19	6-7
Chlorate	2585	1925, 3487	—[c]	—[c]
DBAA	400<LC50<1000	—[c]	212-442	—[c]

BDCM = bromodichloromethane DBAA = dibromoacetic acid

[a] 96 h LC50 = median lethal concentration estimated using probit analysis.

[b] NOEL = no observed (adverse) effect level range calculations from parameter estimates derived using regression analysis.

[c] Not estimable.

Since we were interested in monitoring the effect of this chemical at or near drinking water levels of <0.25 ug/L, no pH adjustment was made to DBAA stocks to extend the test concentration beyond 400 mg/L DBAA. Unfortunately, this concentration was not high enough to provide sufficient mortality for LC50 calculation. The LC50 for DBAA was estimated to be between 400 and 1000 mg/L. Based on changes in fish weight at the different exposure concentrations, a NOEL range (fish weight) of 212-442 mg/L DBAA was determined.

A literature search for comparable research efforts yielded two papers where chloroform acute toxicity was monitored with fish. No papers were found on the other three chemicals used in this study. In studies with the fish, *Poecilia sphenops*, 1/3 males and 0/3 females survived a sixty day exposure to 0.001 mL/L chloroform while 0/3 males and 0/3 females survived the same duration exposure to 0.005 mL/L chloroform (Loekle et al. 1983). The 96 h LC50s for fish exposed to chloroform were: 18.2 mg/L rainbow trout and bluegill; 51.2 mg/L largemouth bass; and 75 mg/L channel catfish (Anderson and Lusty 1980). The chloroform LC50 of 75 mg/L for channel catfish compares to our result of 138 mg/L with medaka. Of the fish tested, medaka are more tolerant.

In summary, medaka acute toxicity was determined with three of the four drinking water disinfection by-products and was estimated for DBAA. The chloroform and BDCM NOEL ranges for both fish weight and length were more than two orders of magnitude higher than median drinking water concentrations of the respective chemicals. The data from this research will be used to determine concentrations for medaka chronic toxicity testing with disinfectant by-products. Medaka sensitivity to the chemicals tested appeared to be ordered from most to least sensitive as

bromodichloromethane>chloroform>dibromoacetic acid>chlorate.

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